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The Impact of Polychlorinated Biphenyls on the Development of Zebrafish (*Danio rerio*)

By

Megan Moma

Accepted in Partial Completion
of the Requirements for the Degree
Master of Science

ADVISORY COMMITTEE

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Master’s Thesis

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Megan Elise Moma

05/17/2023

The Impact of Polychlorinated Biphenyls on the Development of Zebrafish (*Danio rerio*)

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Megan Moma
May 2023

ABSTRACT

Polychlorinated biphenyls (PCBs) are a group of 209 highly stable molecules that were used extensively in industry. Although their commercial use ceased in 1979, they are still present in many aquatic ecosystems due to improper disposal, oceanic currents, atmospheric deposition, and hydrophobic nature. PCBs pose a significant and ongoing threat to the development and sustainability of aquatic organisms. Our hypothesis is that PCB concentration will significantly affect development. Zebrafish (*Danio rerio*) were exposed to a standard PCB mixture (Aroclor 1254) for the first 5 days post fertilization, as there is a gap in knowledge during this important developmental period for fish (i.e., organization of the body). This PCB mixture was formally available commercially and has a high prevalence in PCB contaminated sites. We tested for the effects of PCB dosage on zebrafish survival, rate of metamorphosis, feeding efficiency, and growth. We found significant, dose-dependent effects of PCB exposure on mortality, feeding efficiency, and growth, but did not see a clear effect of PCBs on the rate of zebrafish metamorphosis. Most importantly, we identified a threshold PCB dosage beyond which PCB exposure had a significant impact on life-critical processes. This can further inform local management decisions in environments experiencing PCB contamination.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Jim Cooper, for the reassurance and support throughout my time at Western. Through the countless hours of planning the experiment, to reviewing the results, and guidance with writing, Dr. Cooper was always there to help and offer assistance. I am honored to be able to work alongside and learn from such a dedicated scientist, who was willing to explore a new field and go on this journey with me.

I would also like to thank my committee, Dr. Karin Lemkau and Dr. Brady Olson, for brainstorming, assisting, and strengthening my experiment. Through their expertise, advice, guidance, and support I was able to carry out a successful experiment and become a more well-rounded scientist that I am today. Additionally, I would like to thank Dr. Matthew Zinkgraf for going above and beyond in helping me with my statistical analyses.

A special thank you to the undergraduate students, Abi Lee, Amirah Casey, Marshall Lenhart, and Luke Ghallahorne, who spent hours in the lab assisting with conducting feeding trials, daily fish care, and preparing materials. I want to specifically thank Abi Lee who assisted me every step of the process not only as a friend but also as a fellow scientist aiding in any way she could, I would not be where I am today without her.

Lastly, I would like to thank my family and friends for their constant supportive cheering through reading and rereading my papers, being my test audience for presentations, giving constructive feedback and being my biggest fans. Through their loving support I was able to believe in myself and persevere through the rough patches during my time here at Western. I love you all, thank you again for showing up for me every day and believing in me when I couldn’t.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Supplemental Materials

INTRODUCTION

Polychlorinated biphenyls (PCBs) are a group of 209 highly stable molecules that were first developed in 1929 (Stalling & Mayer, 1972). They were extensively used in machinery such as dielectric fluid capacitors and closed-system heat exchangers as they have a high boiling point, good insulating properties, low flammability, and are chemically stable at high temperatures (Reddy et al., 2019; Stalling & Mayer, 1972). Within the United States, the Monsanto company was the only producer of PCBs. They manufactured eight different commercial preparations trademarked as Aroclors (Stalling & Mayer, 1972). Although sales were restricted to ensure controlled disposal, the massive environmental impact (i.e., high mortality of organisms inhabiting the region) of PCB contamination resulted in the worldwide prohibition of their commercial use in 1979 (Stalling & Mayer, 1972; Hayashi et al., 2015). However, due to their improper disposal, river and ocean currents, atmospheric deposition, and their hydrophobic nature, PCBs are prevalent in many aquatic ecosystems where they pose a significant and ongoing threat (LeRoy et al., 2006; Hashmi et al., 2015; Schwindt, 2015).

PCBs are often described as “legacy contaminants” because of their slow rate of degradation. The prevalence of PCBs in some sediments and their propensity to bioaccumulate makes them a particular threat to both bottom-dwelling species and those that feed at high trophic levels (Foekema et al., 2008; Lovato et al., 2016). Aquatic habitats close to heavily industrialized areas face the highest risk from PCB contamination (Eqani et al., 2013).

Locally, multiple areas within Puget Sound have high levels of PCB contamination (Harrison et al., 1994; McCain et al., 1990; West et al., 2008). Puget Sound is a large fjord system in Washington State that is comprised of numerous, linked inland marine and estuarine areas

(Burns, 1985; Moore et al., 2008). Its relatively restricted water exchange with the Pacific Ocean via the Strait of Juan de Fuca promotes long residence times for aquatic sedimentary pollutants (Harrison et al., 1994).

The bioaccumulation of PCBs in Puget Sound creates significant risks for local species, especially those that feed at high trophic levels in which exposure to PCBs is increased (Hickie et al., 2007). Pacific herring (*Clupea pallasii*) from Puget Sound were reported to have PCB levels 3 to 9 times higher than a nearby herring population in the Strait of Georgia, possibly due to historically higher levels of industrialization in Puget Sound (West et al., 2008). Mean PCB levels in juvenile Chinook salmon (*Oncorhynchus tshawytcha*), a herring predator, from the Duwamish Waterway in Seattle, Washington (an urban estuary in a heavily industrialized area), were three times higher than those of Chinook salmon from the nearby Nisqually River (a site with low levels of PCB contamination; McCain et al., 1990). PCB concentrations within local orca populations that prey heavily on Chinook salmon are also known to reach life-threatening levels (Krahn et al., 2007). These levels have the potential to result in disruptions to immune and endocrine systems, increasing mortality within this endangered species (Krahn et al., 2007). It is estimated that it will take an additional 14-57 years for PCB contamination in the Puget Sound area to fall below levels that pose a risk to local species (Hickie et al., 2007).

Human exposure to PCBs can result in health concerns that range from minor to lethal (e.g., cancer, periorbital edema, gingival hyperplasia, abnormal skull calcification, low birth weight, etc.; Jafarabadi et al., 2019; Ju et al., 2012). The International Agency of Research on Cancer has also deemed PCBs to be a potential carcinogen in humans (Jafarabadi et al., 2019). Washington Department of Health has advocated against human consumption of Chinook Salmon from Puget Sound due to high levels of PCB contamination (Washington Department of Health, 2015).

Consuming contaminated seafood is one of the most likely paths of human PCB exposure, while inhalation and absorption through the skin can also occur (Du et al., 2012; Fattore et al., 2008; Kiviranta et al., 2004; Li et al., 2018; Sirot et al., 2012; Şişman et al., 2007).

In fishes, PCB exposure and subsequent accumulation occurs via two main pathways: prey consumption and uptake via gills, epithelial, and dermal tissues (Antunes & Gil, 2004; Hansen et al., 1971; Mackay & Fraser, 2000; Visha et al., 2018). Once contamination occurs, PCBs can significantly alter development and impair mechanisms of homeostasis (Horri et al., 2018). Many of these effects are the result of PCBs acting as endocrine-disrupting compounds (Horri et al., 2018). PCB exposure can, for example, reduce levels of circulating thyroid hormone (TH) in vertebrates by as much as 30% (Crofton et al., 2005; LeRoy, 2006; Martin et al., 2012; Sumpter et al., 1996).

Fishes are most sensitive to environmental pollutants during early development (Foekema et al., 2012). When exposed to PCBs as embryos, fish are more likely to suffer from long-lasting effects due to the impact that PCBs can have on processes that coordinate anatomical organization, such as TH signaling (Foekema et al., 2012). Adequate TH levels are necessary for fish to metamorphose, build and maintain their skeletons, and develop functional adult feeding mechanisms (Galindo et al., 2019; Keer et al., 2019). Decreased TH levels (hypothyroidism) result in the abnormal retention of cartilaginous regions within the skull vault and incomplete skull ossification, leading to decreased levels of cranial motion that can impair feeding (Galindo et al., 2019; Hu et al., 2019; Keer et al., 2019; McMenamin et al., 2017).

Pollutants stress living systems and can exacerbate the mortality that would normally occur during complicated developmental transitions, such as metamorphosis (Wesner et al., 2014).

Because metamorphosis is associated with high mortality under normal conditions it is sometimes referred to a “bottleneck” period (Wesner et al., 2014). Any toxins that further increase metamorphic mortality can have a large impact on population survival (Wesner et al., 2014).

During development an organism spends a great deal of energy on growth, with a small margin for energy that can be used for other biological processes without impacting survival (Metcalfe & Monaghan, 2001). Metamorphosis requires significant energy expenditure, so that exposure to toxins that interfere with metamorphosis may incur an energetic cost that can lead to mortality (Metcalfe & Monaghan, 2001; Wesner et al., 2014). Within the field of toxicology, it is common for experiments to focus on PCB exposure during early life stages (especially the embryonic period), while ignoring later developmental periods that may be particularly susceptible to PCB toxicity. The high cost of metamorphosis in combination with the fact that PCBs can disrupt the TH signaling that initiates and directs this important developmental transition suggests that this developmental stage could be heavily impacted by PCB exposure.

Further investigations are needed to better understand how PCBs affect development and fitness, especially during later life stages (e.g., metamorphosis and juvenile development). This study provides a better understanding of the effects of PCB contaminants on a model aquatic species, the zebrafish (*Danio rerio*; Hamilton, 1822). Juvenile zebrafish (after metamorphosis, but not sexually mature) exposed to PCBs display muscle dysfunction, swimming defects, disruption of liver metabolism, and decreased reproductive fitness (Hayashi et al., 2015). We complement this study by quantifying the effects of PCB exposure on survival, growth, and feeding efficiency in both pre- and post-metamorphic zebrafish, and by testing for effects on the rate of metamorphosis.

We determined threshold PCB tissue concentrations for significant impacts on the development of young zebrafish and quantified the effects of PCB exposure on their growth, survival, rate of metamorphosis, and feeding ability.

Aroclor 1254 (~21% $C_{12}H_6C_{l4}$, ~48% $C_{12}H_5C_5$, ~23% $C_{12}H_4C_{l6}$, ~6% $C_{12}H_3C_{l7}$) is the commercial PCB mixture that was used in this study (Bast, 1997). It is 54% chlorine by molecular weight (as denoted by the last two digits in its name; Ballschmiter & Zell, 1980; Bast, 1997; Erickson, 1997; Lang, 1992; Parkinson & Safe, 1987). We chose to examine the effects of Aroclor 1254 on zebrafish development because of its high prevalence in PCB-contaminated sites and due to it having been one of the most widely used PCB mixtures (Erickson, 1997). This research will assist conservation biologists and aquatic resource managers with determining when PCB contamination represents a significant risk to fish stocks.

Questions & Hypotheses

Question 1: What PCB concentration significantly affects survival?

Null hypothesis: PCB exposure will not significantly affect survival.

Question 2: What PCB concentrations significantly affect the timing of metamorphosis in fish being exposed?

Null hypothesis: PCB exposure will not significantly affect the timing of metamorphosis.

Question 3: What PCB concentrations significantly affect the feeding efficiency of fish being exposed?

Null hypothesis: PCB exposure will not significantly affect feeding efficiency.

Question 4: What PCB concentrations significantly affect the standard length in fish being exposed?

Null hypothesis: PCB exposure will not significantly affect standard length.

Methods

Fish Breeding, Egg Collection, and Dosing

Wild-type zebrafish (*Danio rerio*; AB line) were used in this study as they are easily bred, have rapid development, and are a model organism with straightforward husbandry (Meyers, 2018). Four male/female zebrafish pairs were placed in each of 4 standard zebrafish breeding tanks (Tecniplast, ZB17BTE; ZB17BTISLOP; ZB17BTL) and maintained at 28 degrees Celsius in an incubator (Shel Lab, SMI6) overnight. Incubator lighting was adjusted to the 14:10 light/dark schedule to which the breeding pairs had been previously acclimated. Fish were placed in tanks after lights out to promote fertilization at artificial sunrise the next day.

After breeding, 60 healthy, fertilized eggs were haphazardly selected and added to each of 42 glass petri plates (90 mm diameter; Bomex, Shanghai, China) containing 50 mL of embryo water (Westerfield, 2000). This allowed for 7 replicates of 6 treatments (plates as replicates).

Because the PCB mixture was dissolved in methanol, we utilized two control treatments: embryo water alone and embryo water plus methanol (a “solvent control”). We will refer to our treatments in the following manner, with the PCB concentrations of the treatment solutions in parentheses: control (embryo water only; 0 mg/L), methanol (solvent control; 0 mg/L); PCB 1 (0.125 mg/L), PCB 2 (0.25 mg/L), PCB 3 (0.35 mg/L), and PCB 4 (0.40 mg/L). All treatments except for the control treatment and PCB 4 received additional methanol so that the concentration of methanol in all treatments (except for the control) were equal. Treatments are reflective of PCB concentrations found within Pacific herring inhabiting Puget Sound (West et al., 2017). Eggs remained in these solutions for 5 days and dead eggs were removed daily. By 5 days-post-fertilization (dpf) all eggs had hatched. Treatment solutions were removed from each plate via pipette and all larvae were gently rinsed three times with embryo water (pipetted carefully into plates and then pipetted out). All dead eggs and PCB water waste were disposed of following an approved animal care protocol (WWU 21-006).

After rinsing, the larvae from 6 plates per treatment (36 plates total) were transferred to individual 4 L mason jars (one jar per plate/replicate) containing 0.50 L of embryo water. Fish from the remaining petri plates (1 per replicate) were euthanized, for initial length comparisons, according to animal care protocol WWU 21-006, fixed in paraformaldehyde and stored as described below. Jars were assigned to one of six, 110-quart plastic tubs using a random number generator in Excel (Microsoft, Inc., Redmond, WA). Water was placed in the bottom of each tub (~3 inches) to provide a water bath. An aquarium heater (Uniclife, HP608) was used to maintain the temperature of the water bath at 28 degrees C and an air stone was used to circulate the heated water throughout the tub and maintain an even temperature throughout. The temperature of the water baths was recorded each day and adjusted as necessary.

Daily Care

Eighty percent of the water in each jar (400 mL) was exchanged for new embryo water every day from 6 dpf onward. PCB wastewater was disposed of according to the approved animal care protocol (WWU 21-006). Ammonia levels were measured and recorded daily for each jar (API NH_3/NH_4^+ Test Kit, API, Chalfont, PA). Ammonia-absorbing sponges (EA Aquatics, San

Rafael, Philippines) were cut into 1.5 cm x 1.5 cm squares added to each jar (1 sponge section per jar). Sponges were replaced and changed every other day. Jars were inspected daily, and any dead fish were removed. Mortality was recorded daily for every jar. The fish in each jar were fed 50 mL of live *Paramecium* culture once daily after water changes and the removal of any dead fish. Beginning at 10 dpf, 3 drops of live, newly hatched brine shrimp (*Artemia*) were also added to each jar using a transfer pipette.

Brine shrimp were raised in standard brine shrimp cones (Brine Shrimp Direct, Ogden, Utah) for 24 hours, then allowed to feed on a commercial algal suspension (Reed Mariculture, Campbell, California) for an additional 24 hours. Cultures were then passed through a brine shrimp strainer (Brine Shrimp Direct, Ogden, Utah), rinsed briefly with deionized water (DI water), and then rinsed from the strainer into a beaker using DI water. Live brine shrimp were allowed to briefly settle to the bottom of the beaker so that concentrated shrimp could be removed by pipette. The amount of shrimp added to each jar daily was gradually increased at a rate that allowed fish to consume all/most shrimp (following established protocols within the lab). Any uneaten shrimp were removed by pipette. On dpf 25 *Paramecia* feeding stopped and fish were only fed 10 drops of brine shrimp once daily for the remainder of the study.

Metamorphosis

Fish were checked daily for signs of metamorphosis starting on 10 dpf (the earliest day at which metamorphosis has been reported in wild-type zebrafish; McMenamin & Parichy, 2013). Fish were first examined in their jars against a solid background in a well-lit area. If any fish appeared to exhibit possible signs of metamorphosis, then the contents of the mason jar were gently decanted into a 2.5 L rectangular tank (Aquaneering Inc., ZT280, San Diego, California) for clearer viewing and confirmation of metamorphosis. Fish were considered to have entered metamorphosis when they exhibited a lateral patch of iridophores (a shiny, white patch of skin) immediately behind the head that was flanked dorsally and ventrally by horizontal lines of melanophores (black lines; Fig. 1; McMenamin & Parichy, 2013). Metamorphosed fish were transferred to a separate mason jar within the same tub and the number of fish that had entered metamorphosis was recorded daily for each of the original jars. Each replicate of every treatment had a dedicated jar for fish that had entered metamorphosis.

Feeding Trials

Feeding trials were performed at 15, 25, and 35 dpf to test for an effect of PCB and/or methanol exposure on feeding proficiency. Five (5) fish were haphazardly collected from each mason jar and placed into a single 250 mL beaker containing 200 mL of embryo water at 28 degrees C. Each beaker was then placed in a lighted incubator at 28 degrees C for ten minutes to allow fish to acclimate. Twenty-five (25) brine shrimp (5 brine shrimp per fish) were then added to each beaker. If there were less than 5 fish alive in a jar, the number of brine shrimp was reduced accordingly to maintain a 5-to-1 shrimp/fish ratio. The water volume in each beaker was also adjusted accordingly. After three (3) minutes ice was added to each beaker to halt feeding and euthanize the fish according to WWU animal care protocol 21-006. The remaining brine shrimp were then counted, and the fish from each beaker were placed in labeled tubes in which they were fixed in a paraformaldehyde solution at 4 degrees C for 24 hours. After fixation fish were slowly transferred into 75% ethanol for storage. The standard length of each fish was measured under a stereomicroscope (Leica Microsystems; Model: MSV269) using digital calipers. All remaining fish were euthanized following the final feeding trial at 35 dpf whether or not they were included in a feeding trial.

Statistical Analyses

Survival

An initial Kaplan-Meier analysis was used to test for differences in survival between treatments. Because no significant difference was found between the control and the solvent treatment (*p*-value= 0.49195; Table S1), the PCB treatments were only compared to the solvent control treatment in subsequent analyses. To take population density into account a Cox-Proportional Hazard analysis (CPH) was used to test for differences in survival between the PCB and methanol treatments. CPH can account for changes in population density over time, whereas Kaplan-Meier analyses cannot. CPH estimates a survival probability for every treatment and then determines the slope of the survival probability (y) PCB concentration (x) relationship. This slope, representing the relationship of the probability of survival for a given PCB treatment, is termed the Hazard Ratio (HR) for survival (Therneau & Grambsch, 2000). A HR was also calculated for population density in order to determine if the number of fish per jar influenced survival. CPH analyses were performed using the 'coxph' function to run a fixed-effects Cox model in the 'survival' package within R Studio (Therneau & Grambsch, 2000; Therneau 2023). The tubs in which the jars were maintained were treated as a random effect and fixed coefficients were calculated to estimate the effects of treatment and population density on survival.

The 'cox.zph' function was used to test the assumption that the Hazard Ratio (HR) was constant throughout the study. A Kaplan-Meier survival plot was used to display daily survival across treatments (Figure 2), with changes in slope indicating when fish deaths occurred (Bland & Altman, 1998).

Metamorphosis

CPH was also used to examine the metamorphosis data. Because the metamorphosis HR for these data was not proportional throughout the study, the 'coxme' function was used to run a mixed-effects Cox model (fixed effects & random effects). This model is not sensitive to the assumption that the HR is constant over time (taking population density into account; Therneau, 2022). Population density was accounted for in this model because population density changed each time metamorphosing fish were removed from their original jar. We used the same random effects within the experimental design as noted above (jars nested within tubs; see '*Survival*' section) and fixed coefficients were also treated in the same manner. HR were calculated for both survival and population density.

Feeding Efficiency

Feeding efficiency was measured as the percentage of available shrimp consumed during a trial (Fig. 4 and 5). A Negative Binomial Model (NBM), which is a specific version of a Generalized Linear Mixed Model (GLMM), was used to compare feeding efficiency across treatments for all feeding trials (15, 25, and 35 dpf). GLMM merges aspects of both a Generalized Linear Model (GLM) and a Mixed Model, and allows for irregular distributions of data (Bolker, 2015). Both fixed and random effects are accounted for within the GLMM model. Fixed effects differentiate differences between treatments. Random effects within this model are the same as those detailed in the section above titled '*Survival.*' An NBM uses a Poisson-Gamma mixture to assess count data and allows for high variance in comparison to the mean (Yirga et al., 2020). This can accommodate overdispersion when the residual variance is higher than what the model can predict.

NBM analyses were run through the ‘lme4’ package in R Studio using the function ‘glmer.nb’ (Bates et al., 2015). The ‘q-q plot’ function in the ‘ggplot’ R package was used to determine if the residuals of the data were normally distributed (an assumption of the NBM; Horikoshi & Tang, 2016; Wickham, 2016).

Pairwise comparisons of feeding efficiency between treatments were then performed for each feeding trial using the package ‘emmeans’ (Lenth et al., 2023). The False Discovery Rate (FDR) correction method (Lenth et al., 2023) was used to adjust *p*-values for multiple comparisons.

Length

A GLMM was used to test for differences in the rate of fish elongation between treatments. The steps of these analyses followed the same order as those described for ‘*Feeding Efficiency*’ above. This model was created using the ‘glmer’ function in the ‘lme4’ package in R Studio (Bates et al., 2015). The function ‘emmeans’ was used to run pairwise comparisons between treatments at each time point (5, 15, 25, and 35 dpf; Lenth et al., 2023). Data from the PCB 4 treatment was not included in the measurements recorded at 35 dpf as all fish in that treatment had died by that time. Q-Q plot was used to verify that the data met the assumptions of the model, and the FDR correction method was also used to adjust *p*-values (see ‘*Feeding Efficiency’* above; Lenth et al., 2023; Wickham, 2016).

RESULTS

Survival

PCB concentration had a significant effect on survival (Table 1). We therefore reject our first null hypothesis; PCB exposure will significantly affect survival. Survival data are visually displayed in Figure 2. At 0 dpf the y-axis is at 1.0 (100% of fish were alive). Neither tub nor jar had a significant effect on survival (Table S2). The HR for survival was significant (p-value < 2e-16) and estimated to be 130.4 (1.304e+02; Table 1), which indicates that exposure to higher PCB concentrations resulted in higher mortality, with PCB treated fish 130 times more likely to die relative to control fish. The HR for population density was significant (p-value= <2e-16) and estimated to be 1.046 (Table 1), indicating that the mortality of PCB-treated fish and control fish were affected in a similar way by population density.

In general, higher rates of mortality were associated with higher PCB dosages (Fig. 2). However, fish in the PCB 2 treatment exhibited survival patterns similar to the control treatment (Fig. 2).

Metamorphosis

A marginally insignificant (p-value= 0.051) effect of PCB concentration on the rate of metamorphosis was found (Table 2). We therefore fail to reject our null hypothesis; PCB exposure will significantly affect the timing of metamorphosis. In general, as PCB concentration increased the rate of metamorphosis decreased (Table 2; Fig. 3). However, an increased rate of metamorphosis was seen in PCB 2 treatment (Fig. 3). The PCB 3 treatment exhibited the slowest rate of metamorphosis (Fig. 3). The PCB 4 treatment was excluded from these analyses as there were only four individuals alive at the onset of metamorphosis and this sample size would not support statistical analysis.

In this analysis an HR of 1 indicates that the treated group acted the same as the solvent control group (methanol) and an HR > 1 indicates a faster rate of metamorphosis relative to the control. An HR of 1.3045 for population density was found to be significant (*p*-value = 0.000; Table 2), which indicates that, across all treatments, the rate of metamorphosis increased as population density within jars decreased. When population density is not accounted for PCB exposure is seen to significantly slow the rate of metamorphosis (HR= 3.621e-07, *p*-value = 4.02e-05; Table 2). However, when population density is included in the model the rate of metamorphosis is marginally insignificant (HR= 0.0316, *p*-value= 0.051; Table 2).

Feeding Efficiency

Fish from all PCB treatments except the PCB 2 treatment had significantly lower feeding efficiencies than control fish at 15 dpf (Table 3, pairwise comparisons between treatments at 15 dpf). No PCB treatments exhibited significantly different feeding efficiency relative to control fish at 25 and 35 dpf (Table 4 and 5; Fig. 4). We therefore cannot reject the null hypothesis that PCB exposure will not significantly affect feeding efficiency. However, except for PCB 2 treatment, we found that PCB exposure affected feeding efficiency in younger, pre-metamorphic fish, but that post-metamorphic fish were not strongly affected. The results of the complete pairwise comparisons are included in the supplementary data (Table S3).

Within each treatment, fish at 15 dpf exhibited significantly lower feeding efficiencies than those at 25 and 35 dpf (Fig. 5). There was no significant difference in the feeding efficiencies of 25 and 35 dpf fish within any treatment (Tables 6-9). Because no fish treated with PCB 4 survived to 35 dpf there could be no comparison with 25 dpf fish from this treatment.

Length

Length distributions were similar across treatments (Table S4; Fig. S1) with one exception. At 35 dpf, the surviving fish that had been treated with the highest PCB concentration at the time (PCB 3) had body lengths that were significantly smaller in comparison to 35 dpf fish from the other treatments (Fig. 6; Table S4). Fish treated with 0.40 mg/L did not survive to 35 dpf. We therefore cannot reject the null hypothesis; PCB exposure will not affect fish length.

Within treatments, 15 dpf fish were significantly longer than 5 dpf specimens, except for those in the methanol treatment (*p*-value= 0.0561; Table 10), and significantly shorter than both 25 and 35 dpf specimens (Tables 10-14; Fig. S1). In both the methanol and PCB 2 treatments fish underwent significant increases in length between each timepoint at which length was measured (i.e., 5, 15, 25 and 35 dpf; Tables 10 and 12). For the PCB 1 and PCB 3 treatments there were no significant differences in the lengths of 25 and 35 dpf fish (Tables 11 and 13). Within the PCB 4 treatment 15 dpf fish were significantly shorter than fish collected at 25 dpf (Table 14).

Among treatments there were no significant differences in the lengths of fishes collected at the same age except for those collected at 35 dpf (Table S4; Fig. 6). At 35 dpf fish from the PCB 3 treatment were significantly shorter than fish from the other treatments in which specimens survived to 35 dpf (i.e., all other treatments except for PCB 4; Table S4; Fig. 6 and S1).

DISCUSSION AND FUTURE DIRECTIONS

We identified a threshold PCB exposure level (0.35 mg/L, i.e., PCB 3; treated from 0-5 dpf), beyond which zebrafish development is substantially impaired in life-critical ways. PCB 3 fish showed exhibited decreased survival, prolonged metamorphosis, decreased feeding efficiency in pre-metamorphic stages, and decreased post-metamorphic growth. PCB 4 fish (0.40 mg/L;

treated from 0-5 dpf) exhibited high mortality from early in the study, did not survive to complete metamorphosis, and had low feeding efficiencies pre-metamorphosis. Identifying this threshold is an important step toward better informing management plans for PCB contaminated sites. If subsequent studies determine the PCB tissue concentration in adult female fishes that can result in their eggs receiving PCB exposure similar to those used in the PCB 3 and 4 treatments here, then aquatic resource managers should be able to better assess environmental threats from PCB toxicity. This study moves a step closer to being able to monitor PCB contamination risks to wild fish stocks by sampling tissues from adult females.

Despite nearly half a century of recovery, PCB exposure continues to be a serious threat to aquatic ecosystems (LeRoy et al., 2006; Hashmi et al., 2015; Schwindt, 2015). Our ability to mitigate the effects of this contamination is limited by our understanding of the threshold PCB exposure levels that produce toxic effects in various species, and the manner in which sub-lethal exposure affects life-critical processes in these organisms. The findings reported here contribute to this understanding by identifying threshold levels of PCB exposure that have lethal effects on a model fish (zebrafish) and by examining how sub-lethal exposure impacts their growth, metamorphosis, and feeding ability.

I examined the effects of exposing zebrafish to the most commonly used commercial PCB mixture: Aroclor 1254 (Erickson, 1997). Aroclor 1254 residues are frequently reported in environmental surveys of PCB contaminated sediments and groundwater (Majone et al., 2015). Although we have some understanding of how exposure to Aroclor 1254 affects vertebrates, this study is the first of its kind to quantify the sub-lethal effects of Aroclor 1254 during later developmental periods (e.g., metamorphosis and early juvenile stages).

Because many PCB toxicity studies have focused on determining lethal PCB contamination thresholds during early development (e.g., Harris et al., 1998; West et al., 2017), it is likely that the impact of these compounds has been underestimated. Aquatic organisms that complete their embryonic and larval stages despite PCB exposure may still experience impairment during later life (West et al., 2017). Reduced growth rates during larval and juvenile development, delayed metamorphosis, and the disorganization of the anatomical remodeling that occurs during metamorphosis, for example, can significantly reduce the survival of aquatic species and heavily impact the annual recruitment of young of the year to existing populations (i.e., stocks; Gilliers et al., 2006; Holzer et al., 2017; Laudet, 2011; West et al., 2017). In addition to quantifying the toxic effects of PCBs on early zebrafish development, our findings also improve our understanding of the threats posed by sub-lethal PCB exposure to the sustainability of wild populations.

Impacts of PCB Exposure on Survival

Many organisms have lower abilities to compensate for toxins (e.g., PCBs) during early developmental relative to later life stages (Crane et al., 2006). Exposure to PCBs can also impact the fundamental body organization that occurs during embryogenesis (Yang et al., 2016). Because early development is so sensitive to PCB toxicity, we exposed specimens to Aroclor 1254 immediately after fertilization in order to document the most severe effects of this compound on developing zebrafish.

We found that PCB exposure during early development significantly affected survival throughout embryonic, larval, and early juvenile life stages (Table 1; Fig. 2). PCB treated fish were 130 times more likely to die than methanol treated fish, with the chance of mortality increasing with PCB concentration (Table 1). These results were consistent with those from similar studies of the effects of PCBs on fishes (Ju et al., 2012; Billsson et al., 1998).

There was higher mortality in early development (0-15 dpf), when fish are generally more susceptible to the effects of PCBs (Schimmel et al., 1974; Fig. 2). With the exception of the PCB 2 treatment, we also see a dose-dependent effect of PCB exposure (the strength of the PCB concentration in which fish were immersed) on survival (Fig. 2). Most notably, some specimens from every treatment lived until 25 dpf, when zebrafish are normally metamorphosing (Guerrera et al., 2015). The PCB concentrations to which we exposed our specimens were therefore low enough that they did not necessarily prevent zebrafish from reaching the age at which, under normal conditions, they would have completed larval development (i.e., entered metamorphosis).

One of the more important findings of this study is the identification of a threshold PCB exposure concentration for completing fish metamorphosis. In each PCB treatment except PCB 4 we saw specimens that were able to complete metamorphosis and live until 35 dpf (Fig. 2). This suggests that exposure to PCB concentrations between 0.35 and 0.40 mg/L (a very narrow range) prevents zebrafish from completing metamorphosis, which is already a developmental transition associated with high mortality in wild fishes (Barth et al., 2015; Campero et al., 2008; Dixson et al., 2011; Doherty, 2002; Dufour & Galzin, 1993; Leis & McCormick, 2002; McCormick et al., 2002; Wesner et al., 2014).

Previous studies that identified higher thresholds for lethal effects of PCBs largely examined embryonic development alone (e.g., Bergeron et al., 1994; Nakayama et al., 2005; Singleman et al., 2021). Because we also examined later developmental periods, we are able to provide a more accurate estimate of the PCB exposure levels that affect the mortality of developing fishes. This information provides aquatic resource managers with a more accurate threshold for PCB concentrations that will impair the sustainability of wild stocks.

Because PCBs can undergo maternal transfer to eggs and young, fishes that develop in PCB-free environments may still be affected by these toxins if their mothers were previously exposed (Foekema et al., 2008). This occurs as PCBs are transferred to the egg with lipids and proteins (Foekema et al., 2008; Nakayama et al., 2005; Daley et al., 2009; Debruyn et al., 2004; Kelly et al., 2011; Russell et al., 1999; Serrano et al., 2008). Additionally, maternally inherited pollutants are rarely excreted, and reach peak concentration during the final stages of the yolk-sac embryonic stage of an individual's life cycle, which can lead to significant developmental disruptions (Foekema et al., 2012).

Threats of PCB contamination to fish stocks are frequently estimated by measuring the PCB concentrations present in the tissues of adult fishes. Our findings suggest that maternal tissue concentrations above 0.35 mg/L (PCB 3) could prevent offspring from surviving to the juvenile stage (Fig. 2 & 3; Foekema et al., 2012; Russell et al., 1999). The results of this study therefore provide aquatic resource managers with a useful threshold value that can improve their ability to use current sampling methods (measure of PCB tissue concentrations in adult fishes) to determine if fish stocks are at risk from PCB contamination.

Impacts of PCB Exposure on Metamorphosis

An individual's survival of metamorphosis is heavily influenced by their energy reserves (Olivotto et al., 2011). Metamorphosis is typically an important "bottleneck period" where post-metamorphic survivors frequently represent a small fraction of the original population (Barth et al., 2015; Campero et al., 2008; Dixson et al., 2011; Doherty, 2002; Dufour & Galzin, 1993; Leis

& McCormick, 2002; McCormick et al., 2002; Wesner et al., 2014). Any toxins that impair such a sensitive developmental transition are of strong interest to aquatic resource managers.

PCB exposure has been linked to decreased levels of TH, which plays a significant role in instigating and directing vertebrate metamorphosis (Dong et al., 2014; LeRoy et al., 2006). Organisms that are unable to maintain satisfactory TH levels may undergo metamorphic delays and/or impairment of the anatomical remodeling that occurs during metamorphosis (Cooper et al., 2020; Galindo et al. 2019; Hodin, 2006; Truman & Riddiford, 1999). Metamorphosis is also physiologically demanding (Campero et al., 2008; Heyland & Moroz, 2006; McCauley et al., 2011; Menon & Rozman, 2007; Wesner et al., 2020). The stress associated with metamorphosis can be exacerbated in polluted environments (Hodin, 2006) because many toxins impair this important developmental transition (Campero et al., 2008; Debecker et al., 2017; Hodin, 2006; Wesner et al., 2014). The presence of Aroclor 1254 in aquatic environments has been shown to prolong the metamorphosis of resident species (Dong et al., 2014; LeRoy et al., 2006; Eales & Brown, 1993; Glennemeier & Denver, 2001; Werner, 1986; Wilbur, 1980). Such elongation of metamorphosis has been shown to increase mortality (McCarthy et al., 2003).

Those organisms that survive larval development after PCB exposure may experience prolonged and/or disrupted metamorphosis (Billsson et al., 1998; Campero et al., 2008; Dong et al., 2014; Grafe et al., 2004; Schimmel et al., 1974). The metamorphic transition from larva to juvenile is characterized by major transformations in feeding behavior and feeding mechanics (McMenamin et al., 2017). Significant impacts on the development of post larval feeding mechanics can therefore result from TH disruption (Galindo et al. 2019; Cooper et al., 2020; McMenamin et al., 2017).

PCB exposure delayed metamorphosis in a concentration-dependent manner (HR= 3.621e-07, *p*-value= 4.02e-05; Table 2; Fig. 3). Population density was also found to inversely affect the rate of metamorphosis. When there were less fish in a jar metamorphosis occurred more quickly (HR = 1.3045, *p*-value= 0.000; Table 2). Even though lower population densities significantly increased rates of metamorphosis (*p*-value<0.0001, Table 2), when population density was taken into account the *p*-value for the effect of PCBs on the rate of metamorphosis was still 0.051 (Table 2). Methanol and PCB 1 treatments exhibited the most rapid metamorphic rate initially, but this rate leveled off for the remainder of the experiment. PCB 3 showed the slowest metamorphic rate (Table 2; Fig. 3). The metamorphosis of fish treated with PCB 2 departed from the pattern established within the other treatments. These fish initially exhibited a rapid rate of metamorphosis and had more individuals entering metamorphosis than in the other treatments (Fig. 3).

Although we found that zebrafish exposed to PCB concentrations greater than 0.35 mg/L (PCB 3) did not complete metamorphosis, we did not find a significant effect of PCB concentration on the rate at which the remaining treatments entered metamorphosis (Table 2; Fig. 3). This may be due to errors in dosing those specimens treated with PCB 2, as the fish in that treatment showed a markedly different response to PCBs than those in other treatments (Table 2; Fig. 3). Visual comparisons of the rates of metamorphosis exhibited by the methanol, PCB 1, and PCB 3 treatments suggest a dose-dependent effect of PCB concentration on metamorphic rate that would likely place wild populations at risk (Table 2; Fig. 3; McCarthy et al., 2003).

Impacts of PCB Exposure on Feeding Efficiency

PCBs affected the feeding efficiency of pre-metamorphic zebrafish larvae (15 dpf) in a concentration-dependent manner, but we saw no effects from PCBs on juvenile (i.e., post-metamorphic) fish at 25 and 35 dpf (Tables 3-5). When examining each treatment individually, feeding efficiency was found to be significantly lower at 15 dpf (pre-metamorphic fish) in comparison to 25 and 35 dpf (post-metamorphic fish; Table 6-9). This supports the conclusion that PCB exposure has a higher impact on larval feeding efficiency than it does on post-metamorphic feeding. Death from larval starvation is common in fishes, and reduced larval feeding can have a strong impact on the seasonal recruitment of young fishes to existing populations (Cushing, 1990; Holzer et al., 2017; Kasumyan, 2001; Leaf & Friedland, 2014; Lusseau et al., 2014; Pritt et al., 2014). Even larval fishes that feed sufficiently to survive metamorphosis may experience decreased growth, reproduction, and survival in later life if larval feeding was impaired (Gilliers et al., 2006; West et al., 2017).

Impacts of PCB Exposure on Growth

We saw no effects of PCB exposure on growth until after metamorphosis, when the fish in the PCB 3 treatment, which was the treatment with the highest PCB dosage in which any fish survived past metamorphosis, were found to be significantly shorter than fish from the other treatments (Fig. 6; Table S4). There was also a difference in post-metamorphic growth between treatments. Fish in the methanol and PCB 2 treatments exhibited significant growth between days 25 and 35, while PCB 1 and PCB 3 fish showed no significant elongation during this time (Tables 10-13). Fish in the methanol and PCB 4 treatments showed no significant elongation between 5 and 15 dpf (Tables 10, 14).

Disruptions in TH signaling are known to retard the growth of young fishes and PCB exposure can reduce TH levels (Dong et al., 2014). The possible correspondence between slower growth and higher PCB exposure could therefore be the result of lower TH levels in fish treated with higher PCB doses. It should, however, be noted that PCBs affect multiple aspects of development and that these different disruptions could have negative additive effects on elongation (Gutleb et al., 1999; Kimbrough & Krouska, 2003; Lundberg et al., 2006; Schmidt et al., 2005; Singleman et al., 2021; Sisman et al., 2007; Ulbrich & Stahlmann, 2004). We cannot therefore definitively attribute the growth reductions seen here to the impact of PCBs on TH signaling alone.

Limitations

The greatest limitation of a toxicological laboratory experiment is the inability to represent what organisms will encounter in the wild. In a natural setting it is highly unlikely that organisms will face only a single environmental stressor (e.g., PCB exposure). Wild populations utilize a wide range of resources that are often limited and insufficient for the survival of all members. Additional stress is added to a population with fluctuations in temperature, pH, and other environmental variables, as well as being exposed to multiple pollutants. The interaction of these variables is likely to have complex effects on fish development that will be difficult to predict.

Another limitation is inconsistency in the composition of Aroclor 1254 mixtures. The ratio of individual PCB congeners found within Aroclor mixtures varies across batches (Johnson et al., 2015; Kodavanti et al., 2001). Because of the various compositions of these mixtures, it is difficult to compare studies in which different vials were used (Burgin et al., 2001; Kodavanti et al., 2001; LeRoy et al., 2006).

Future Directions

Survival, the rate of metamorphosis, feeding efficiency, and the rate of growth in the PCB 2 fish were at odds with observations of fish from the other treatments (Tables 3, 8, and 12; Figs. 2, 3, and 6). This suggests that there may have been a dosing error when setting up the PCB 2 treatment. We suspect that PCB dosage has a significant effect on post-metamorphic growth (i.e., that the PCB 2 results may be in error) but cannot conclude that this is accurate without replicating at least some aspects of this study. Currently, the methanol and PCB 2 treatments of this experiment are being replicated by two undergraduates (Luke Ghallahorne and Marshall Lenhart) under the supervision of Megan Moma and Jim Cooper. The findings of this ongoing study will allow us to determine how to move forward with publishing our combined findings.

If exposure to Aroclor 1254 does affect the growth of post-metamorphic fishes, then this strengthens the case for expanding the scope of PCB toxicology studies. Such work has heavily emphasized testing for the effects of PCB exposure using data from early developmental stages (Foekema et al., 2012). By failing to examine the effects of sub-lethal PCB doses on late development (late larval and juvenile stages) these studies may have overlooked important aspects of how these pollutants impact wild populations. Through additional studies we can create better management plans in order to conserve these ecosystems and the organisms inhabiting them.

TABLES

Table 1. Results of Cox Proportional Hazard Analyses of Survival Data. The estimated effects of PCB concentration and population density on survival are reported. COEF: Estimated Coefficient, quantifies the effect of each covariate on the Hazard Ratio (HR), EXP(COEF): HR, SE(COEF): Standard Error of COEF, Z: Assesses statistical significance of the COEF, PR(>|Z|): *p*-value.

	COEF	EXP(COEF)	SE(COEF)	Z	PR(>|Z|)
PCB	4.870e+00	1.304e+02	2.817e-01	17.29	**<2e-16**
POPULATION	4.457e-02	1.046e+00	1.181e-03	37.73	**<2e-16**

Table 2. Results of a Mixed Effects Cox Proportional Hazard Analyses of Metamorphosis Data (with and without population density taken into account). The estimated effects of PCB concentration and population density on the timing of metamorphosis are reported. COEF: Estimated Coefficient, quantifies the effect of each covariate on the Hazard Ratio (HR), EXP(COEF): HR, SE(COEF): Standard Error of COEF, Z: Assesses statistical significance of the COEF, PR(>|Z|): *p*-value.

	COEF	EXP(COEF)	SE(COEF)	Z	PR(>|Z|)
WITHOUT ACCOUNTING FOR POPULATION DENSITY					
PCB	-3.454	0.0316	1.7701	-1.95	0.051
POPULATION	0.2658	1.3045	0.0193	13.77	**0.000**
WITH ACCOUNTING FOR POPULATION DENSITY					
PCB	1.483e+01	3.621e-07	3.612e+00	-4.106	**4.02e-05**
POPULATION	NA	NA	0	NA	NA

Table 3. Results of 'Emmeans' Analyses of Feeding Efficiency Data (15 dpf). FDR-corrected *p*-values are provided for each comparison.

	PCB 1	PCB 2	PCB 3	PCB 4
PCB 2	0.0870	-	-	-
PCB 3	1.0000	0.1170	-	-
PCB 4	0.7255	**0.0311**	0.6585	-
METHANOL	**0.0067**	0.6247	**0.0099**	**0.0028**

Table 4. Results of 'Emmeans' Analyses of Feeding Efficiency Data (25 dpf). FDR-corrected *p*-values are provided for each comparison. All values > 0.05 therefore no significant difference in feeding efficiency.

	PCB 1	PCB 2	PCB 3	PCB 4
PCB 2	0.3429	-	-	-
PCB 3	0.8026	0.7142	-	-
PCB 4	0.1516	0.5048	0.2979	-
METHANOL	0.8174	0.6247	1.0000	0.2533

Table 5. Results of 'Emmeans' Analyses of Feeding Efficiency Data (35 dpf). FDR -corrected *p*-values are provided for each comparison. PCB 4 was excluded as no individuals from this treatment lived to 35 dpf.

	PCB 1	PCB 2	PCB 3
PCB 2	0.7376	-	-
PCB 3	0.3770	0.6247	-
METHANOL	0.8174	1.0000	0.5803

Table 6. Results of 'Emmeans' Analyses of Feeding Efficiency Data from the Methanol Treatment Across Feeding Trials. FDR-corrected *p*-values are provided for each comparison.

	METHANOL 15 DPF	METHANOL 25 DPF
METHANOL 25 DPF	**0.0003**	-
METHANOL 35 DPF	**0.0003**	1.0000

Table 7. Results of 'Emmeans' Analyses of Feeding Efficiency Data from the PCB 1 Treatment Across Feeding Trials. FDR-corrected *p*-values are provided for each comparison.

	PCB 1 (15 DPF)	PCB 1 (25 DPF)
PCB 1 (25 DPF)	**<0.0001**	-
PCB 1 (35 DPF)	**<0.0001**	1.0000

Table 8. Table 8. Results of 'Emmeans' Analyses of Feeding Efficiency Data from the PCB 2 Treatment Across Feeding Trials. FDR-corrected *p*-values are provided for each comparison.

	PCB 2 (15 DPF)	PCB 2 (25 DPF)
PCB 2 (25 DPF)	**0.0003**	-
PCB 2 (35 DPF)	**<0.0001**	0.6585

Table 9. Results of 'Emmeans' Analyses of Feeding Efficiency Data from the PCB 3 Treatment Across Feeding Trials. FDR-corrected *p*-values are provided for each comparison.

	PCB 3 (15 DPF)	PCB 3 (25 DPF)
PCB 3 (25 DPF)	**<0.0001**	-
PCB 3 (35 DPF)	**0.0003**	0.6432

Table 10. Results of 'Emmeans' Analyses of Length Data from the Methanol Treatment. FDR-corrected *p*-values are provided for each comparison.

	METHANOL 5 DPF	METHANOL 15 DPF	METHANOL 25 DPF
METHANOL 15 DPF	0.0561	-	
METHANOL 25 DPF	**<0.001**	**<0.001**	-
METHANOL 35 DPF	**<0.001**	**<0.001**	**<0.001**

Table 11. Results of 'Emmeans' Analyses of Length Data from the PCB 1 Treatment. FDR-corrected *p*-values are provided for each comparison.

	PCB 1 (5 DPF)	PCB 1 (15 DPF)	PCB 1 (25 DPF)
PCB 1 (15 DPF)	**0.0072**	-	
PCB 1 (25 DPF)	**<0.001**	**<0.001**	-
PCB 1 (35 DPF)	**<0.001**	**<0.001**	0.0874

Table 12. Results of 'Emmeans' Analyses of Length Data from the PCB 2 Treatment. FDR-corrected *p*-values are provided for each comparison.

	PCB 2 (5 DPF)	PCB 2 (15 DPF)	PCB 2 (25 DPF)
PCB 2 (15 DPF)	**0.0048**	-	
PCB 2 (25 DPF)	**<0.001**	**<0.001**	-
PCB 2 (35 DPF)	**<0.001**	**<0.001**	**<0.001**

Table 13. Results of 'Emmeans' Analyses of Length Data from the PCB 3 Treatment. FDR-corrected *p*-values are provided for each comparison.

	PCB 3 (5 DPF)	PCB 3 (15 DPF)	PCB 3 (25 DPF)
PCB 3 (15 DPF)	**0.0031**	-	
PCB 3 (25 DPF)	**<0.001**	**<0.001**	-
PCB 3 (35 DPF)	**<0.001**	**<0.001**	0.8503

Table 14. Results of 'Emmeans' Analyses of Length Data from the PCB 4 Treatment. FDR-corrected *p*-values are provided for each comparison.

	PCB 4 (5 DPF)	PCB 4 (15 DPF)	PCB 4 (25 DPF)
PCB 4 (15 DPF)	0.1124	-	
PCB 4 (25 DPF)	**<0.001**	**<0.001**	-

FIGURES

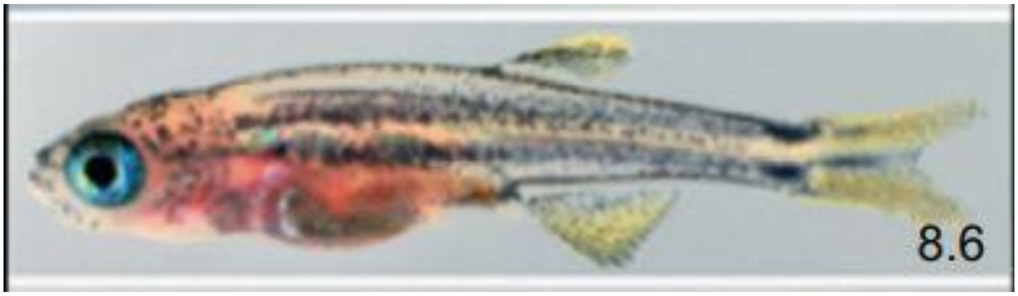

Figure 1. Representative Image of a Zebrafish that has Entered Metamorphosis (image from McMenamin & Parichy, 2013; fish 8.6 mm in length). The appearance of two lateral black stripes bounding a patch of iridophores (white/reflective) behind the head was considered diagnostic of metamorphosing fish.

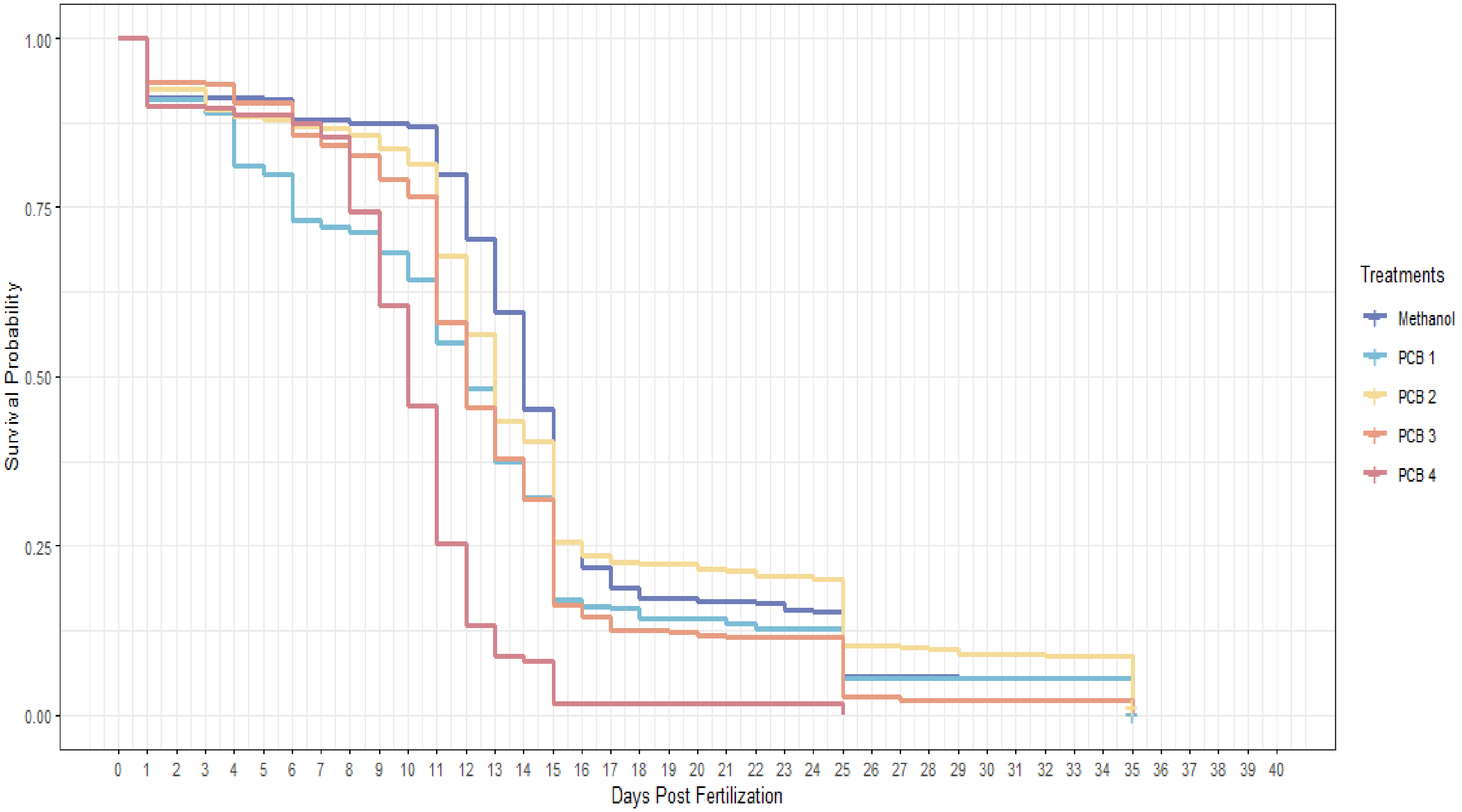

Figure 2. Kaplan Meier Survival Plot Detailing Fish Survival in this Study. Horizontal hash marks at 35 dpf denote individuals that did not die because of PCB exposure but were sacrificed at the end of the experiment.

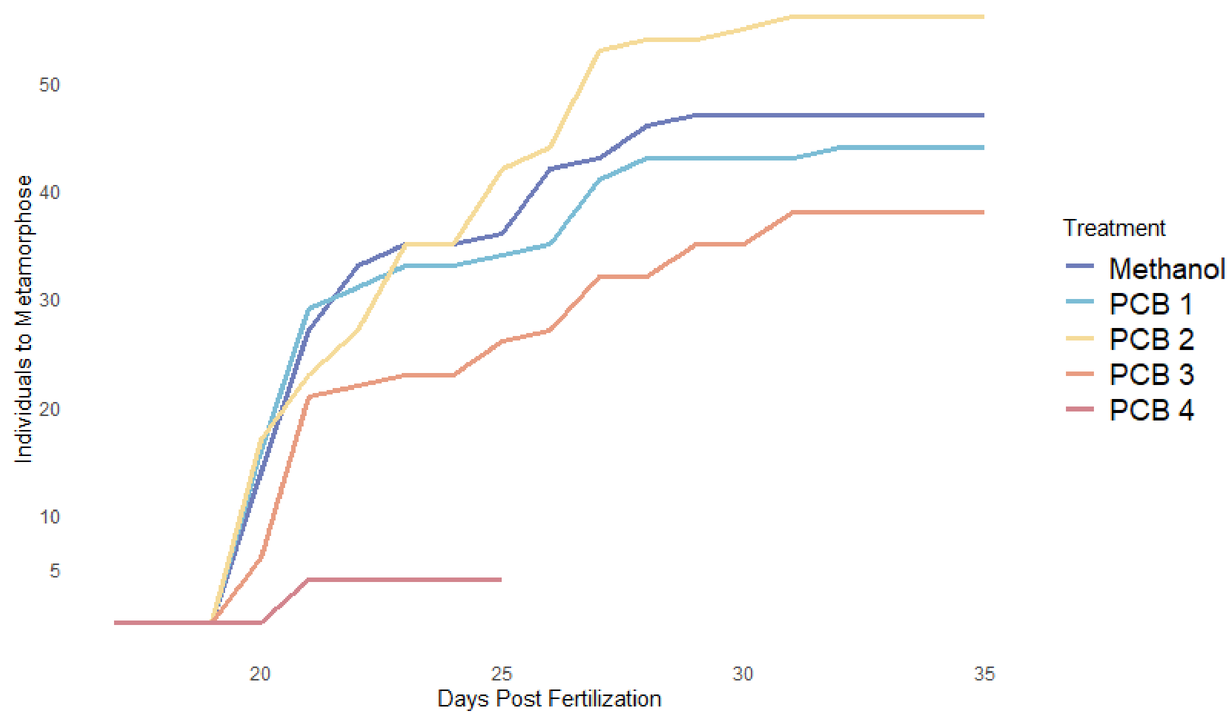

Figure 3. Metamorphosis Across Treatments.

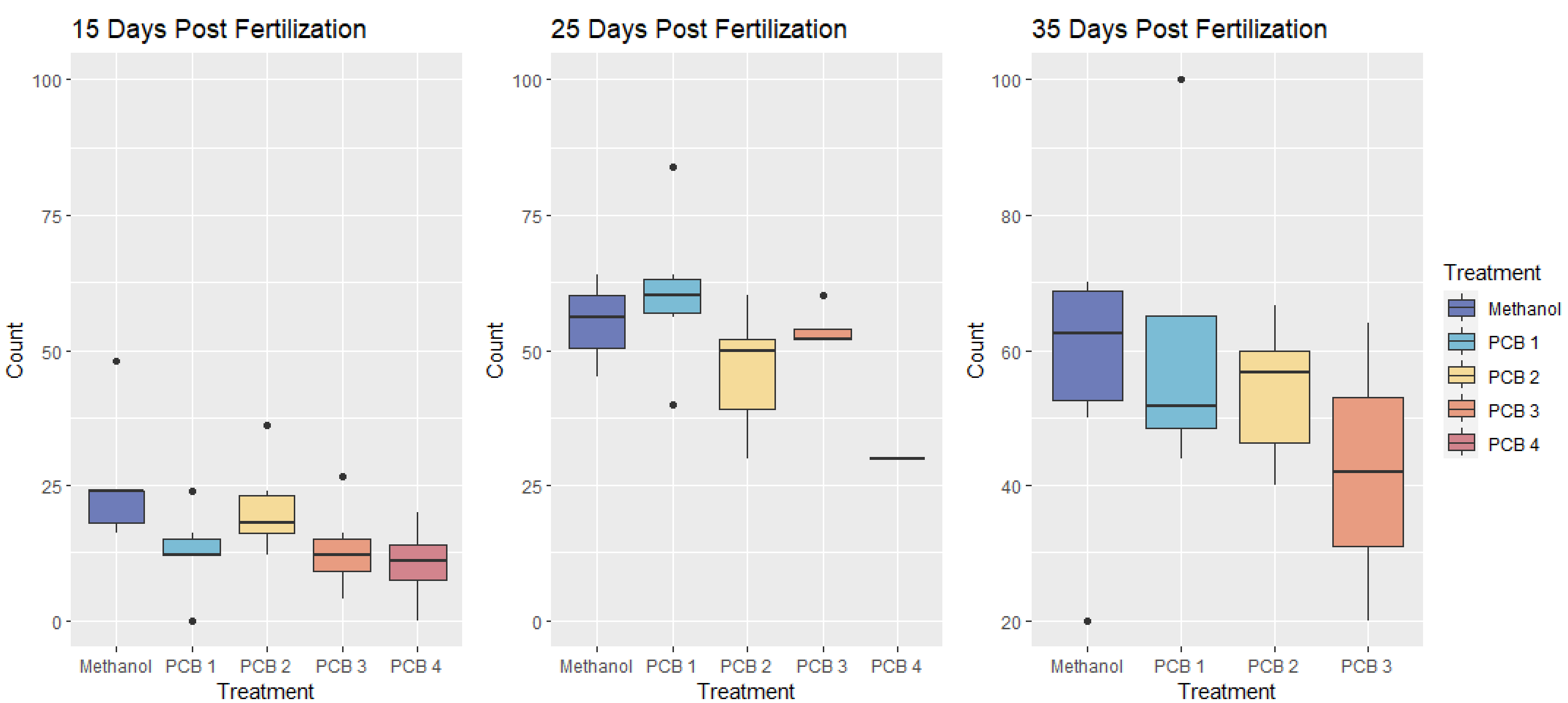

Figure 4. Comparative Feeding Efficiencies of Treatments at 15-, 25-, and 35-Days Post Fertilization (DPF). No PCB 4 fish survived until 35 DPF.

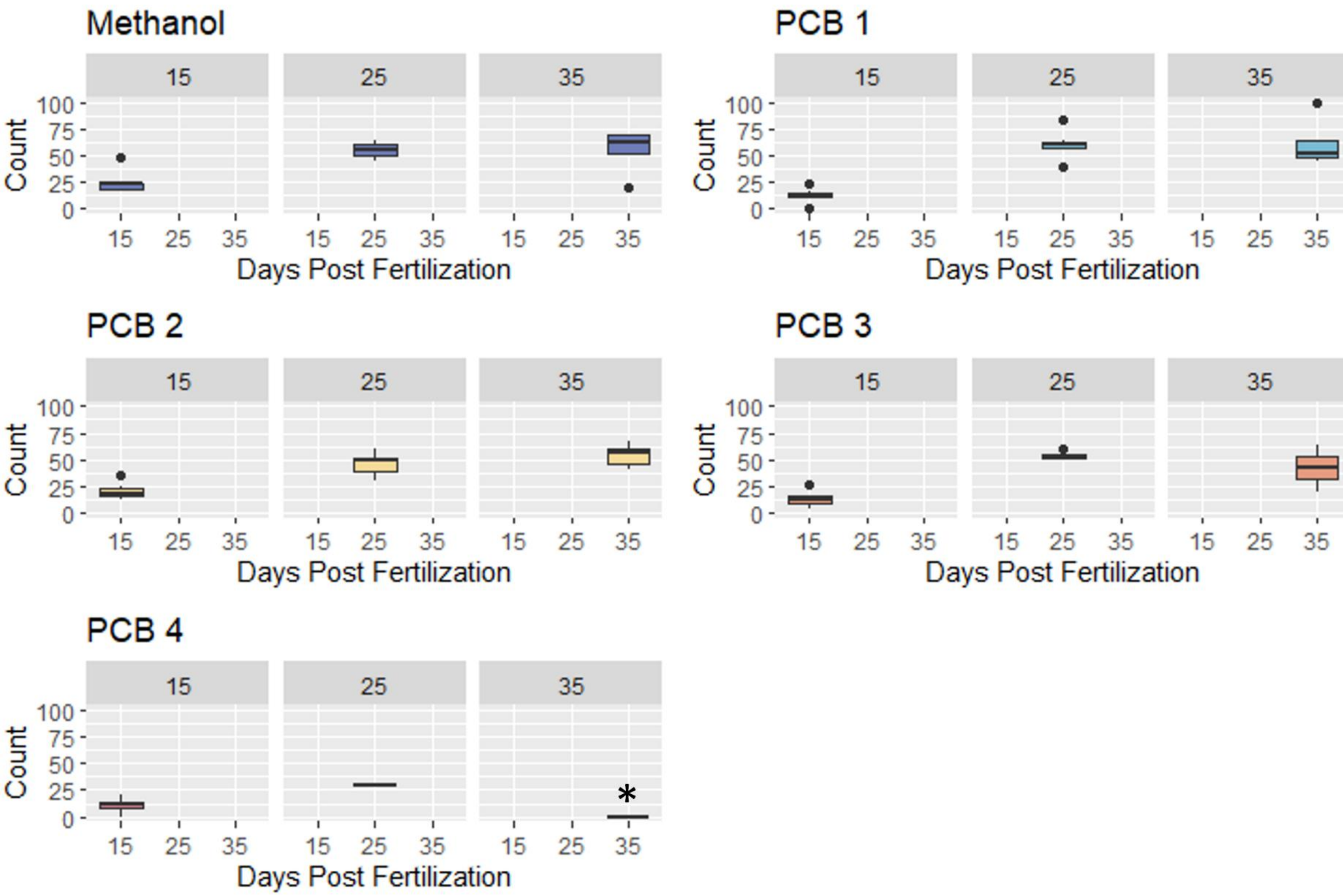

Figure 5. Comparative Feeding Efficiencies within Treatments and Across Development.
(* No PCB 4 fish survived until 35 Days Post Fertilization (DPF).

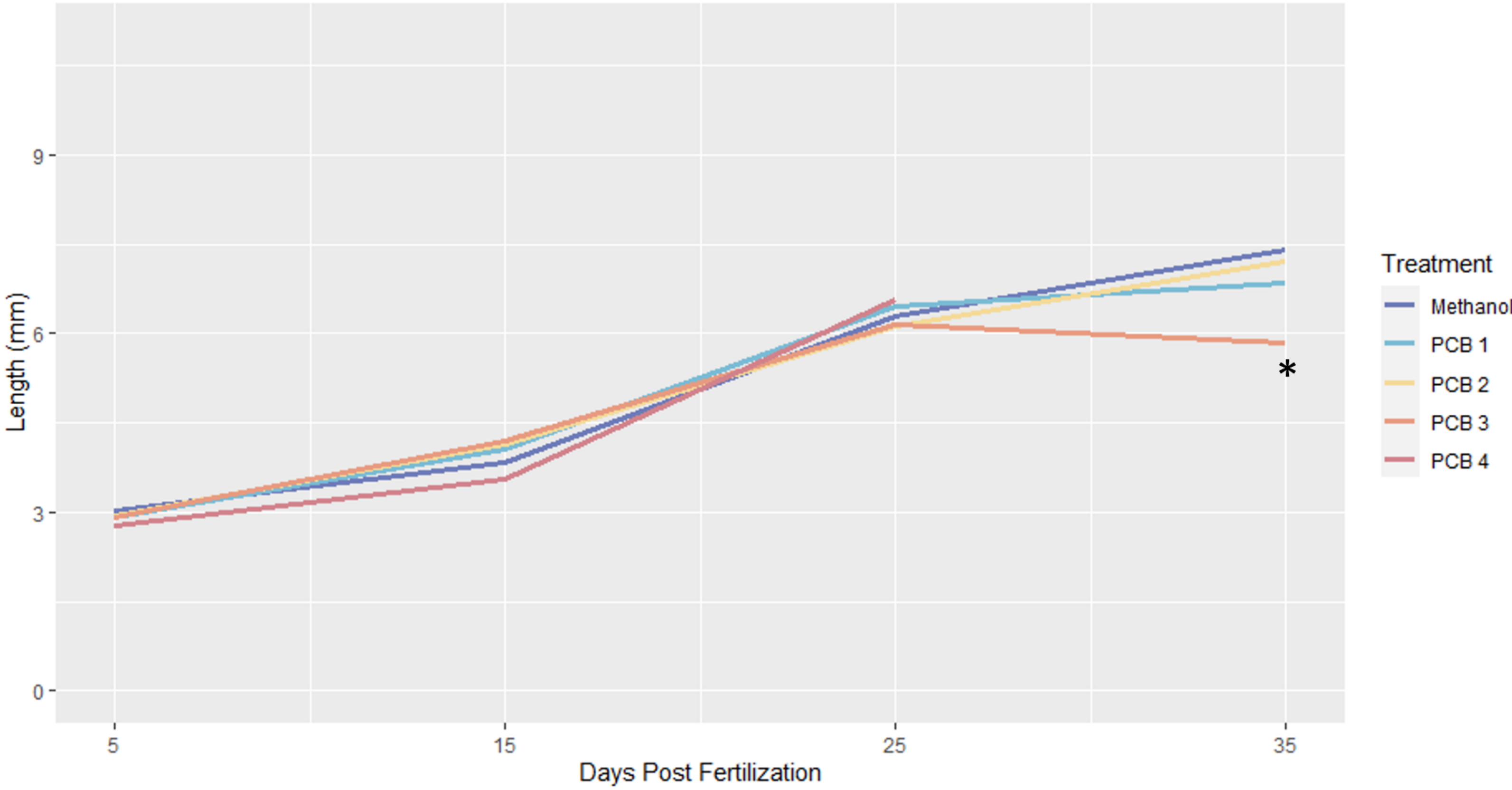

Figure 6. Comparative Average Lengths Across Treatments. No PCB 4 fish survived until 35 Days Post Fertilization (DPF). (***** PCB 3 was significantly shorter than other treatments at 35 DPF). See Figure S1 for individual treatment length distributions.

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SUPPLEMENTARY MATERIALS

Table S1. Results of 'Emmeans' Analyses of Survival Data. BH-corrected *p*-values are provided for each comparison.

	0.125 MG/L	0.25 MG/L	0.35 MG/L	0.40 MG/L	CONTROL
0.25 MG/L	**0.00242**	-	-	-	-
0.35 MG/L	0.96507	**0.00069**	-	-	-
0.40 MG/L	**1.80E-12**	**< 2E-16**	**< 2E-16**	-	-
CONTROL	**0.00012**	0.39763	**2.90E-05**	**<2E-16**	-
METHANOL	**0.00069**	0.89072	**9.10E-05**	**<2E-16**	0.49195

Table S2. Results of a Mixed Effects Cox Proportional Hazard Analyses of Survival. The estimated effects of PCB concentration and population density on survival are reported. Random effects Tank/Jar were not found to be significant (Random Effects: Std Dev. = 1.4153, Variance= 2.0032). COEF: Estimated Coefficient, quantifies the effect of each covariate on the Hazard Ratio (HR), EXP(COEF): HR, SE(COEF): Standard Error of COEF, Z: Assesses statistical significance of the COEF, PR(>|Z|): *p*-value.

	COEF	EXP(COEF)	SE(COEF)	Z	PR(>|Z|)
PCB	9.49222	13256.2	2.23390	4.25	**2.1e-05**
POP	0.07610	1.07901	0.00202	37.66	**0.0+00**

Table S3. Results (Full) of 'Emmeans' Analyses of Feeding Efficiency Data. FDR-corrected *p*-values are provided for each comparison. PCB 4 was excluded from 35 DPF as there were no individuals alive.

TREATMENT	DPF	CONTRAST	ESTIMATE	SE	DF	Z-RATIO	*P*-VALUE
Methanol	-	DPF15-DPF25	-0.7782	0.193	INF	-4.023	**0.0003**
Methanol	-	DPF15-DPF35	-0.7903	0.193	INF	-4.087	**0.0003**
Methanol	-	DPF25-DPF35	-0.0120	0.184	INF	-0.065	1.0000
PCB 1	-	DPF15-DPF25	-1.5664	0.209	INF	-7.491	**<0.0001**
PCB 1	-	DPF15-DPF35	-1.5855	0.228	INF	-6.954	**<0.0001**
PCB 1	-	DPF25-DPF35	-0.0190	0.204	INF	-0.093	1.0000
PCB 2	-	DPF15-DPF25	-0.8073	0.199	INF	-4.063	**0.0003**
PCB 2	-	DPF15-DPF35	-0.9605	0.197	INF	-4.865	**<0.0001**
PCB 2	-	DPF25-DPF35	-0.1531	0.186	INF	-0.824	0.6585
PCB 3	-	DPF15-DPF25	-1.4155	0.228	INF	-6.201	**<0.0001**
PCB 3	-	DPF15-DPF35	-1.1642	0.283	INF	-4.109	**0.0003**
PCB 3	-	DPF25-DPF35	0.2513	0.281	INF	0.893	0.6432
PCB 4	-	DPF15-DPF25	-1.0498	0.402	INF	-2.613	**0.0311**
-	15	Methanol- PCB 1	0.6931	0.218	INF	3.178	**0.0067**
-	15	Methanol-PCB 2	0.2036	0.206	INF	0.988	0.6247
-	15	Methanol-PCB 3	0.6587	0.217	INF	3.035	**0.0099**
-	15	Methanol-PCB 4	0.8807	0.255	INF	6.450	**0.0028**
-	15	PCB 1 – PCB 2	-0.4895	0.221	INF	-2.211	0.0870
-	15	PCB 1 – PCB 3	-0.0345	0.232	INF	-0.149	1.0000
-	15	PCB 1 – PCB 4	0.1876	0.268	INF	0.700	0.7255
-	15	PCB 2 – PCB 3	0.4551	0.220	INF	2.064	0.1170
-	15	PCB 2 – PCB 4	0.6771	0.258	INF	2.623	**0.0311**
-	15	PCB 3 – PCB 4	0.2221	0.267	INF	0.832	0.6585
-	25	Methanol-PCB 1	-0.0950	0.183	INF	-0.519	0.8174
-	25	Methanol-PCB 2	0.1745	0.186	INF	0.940	0.6247
-	25	Methanol-PCB 3	0.0214	0.206	INF	0.104	1.0000
-	25	Methanol-PCB 4	0.6092	0.366	INF	1.666	0.2535
-	25	PCB 1 – PCB 2	0.2695	0.185	INF	1.458	0.3429
-	25	PCB 1 – PCB 3	0.1164	0.205	INF	0.567	0.8026
-	25	PCB 1 – PCB 4	0.7042	0.365	INF	1.928	0.1516
-	25	PCB 2 – PCB 3	-0.1531	0.207	INF	-0.738	0.7142
-	25	PCB 2 – PCB 4	0.4347	0.366	INF	1.186	0.5048
-	25	PCB 3 – PCB 4	0.5878	0.377	INF	1.558	0.2979
-	35	Methanol-PCB 1	-0.1021	0.204	INF	-0.499	0.8174
-	35	Methanol-PCB 2	0.0334	0.184	INF	0.181	1.0000
-	35	Methanol-PCB 3	0.2847	0.266	INF	1.072	0.5803
-	35	PCB 1 – PCB 2	0.1355	0.205	INF	0.662	0.7376
-	35	PCB 1 – PCB 3	0.3868	0.280	INF	1.380	0.3770
-	35	PCB 2 – PCB 3	0.2513	0.266	INF	0.946	0.6247

Table S4. Results (Full) of 'Emmeans' Analyses of Length Data. FDR-corrected *p*-values are provided for each comparison. PCB 4 was excluded from 35 DPF as there were no individuals alive.

TREATMENT	DPF	CONTRAST	ESTIMATE	SE	DF	T-RATIO	*P*-VALUE
Methanol	.	DPF5 - DPF15	-0.8046	0.313	14.8	-2.567	0.0561
Methanol	.	DPF5 - DPF25	-3.2672	0.315	15.1	-10.359	**<0.001**
Methanol	.	DPF5 - DPF35	-4.4178	0.338	19.5	-13.089	**<0.001**
Methanol	.	DPF15 - DPF25	-2.4626	0.185	386.8	-13.319	**<0.001**
Methanol	.	DPF15 - DPF35	-3.6132	0.221	398.2	-16.385	**<0.001**
Methanol	.	DPF25 - DPF35	-1.1506	0.223	397.9	-5.156	**<0.001**
PCB 1	.	DPF5 - DPF15	-1.1344	0.313	14.8	-3.619	**0.0072**
PCB 1	.	DPF5 - DPF25	-3.5902	0.325	16.7	-11.034	**<0.001**
PCB 1	.	DPF5 - DPF35	-4.0744	0.339	18.9	-12.003	**<0.001**
PCB 1	.	DPF15 - DPF25	-2.4558	0.201	405.6	-12.191	**<0.001**
PCB 1	.	DPF15 - DPF35	-2.9400	0.223	401.8	-13.158	**<0.001**
PCB 1	.	DPF25 - DPF35	-0.4841	0.230	398.8	-2.102	0.0874
PCB 2	.	DPF5 - DPF15	-1.2051	0.313	14.8	-3.845	**0.0048**
PCB 2	.	DPF5 - DPF25	-3.1935	0.314	15.0	-10.157	**<0.001**
PCB 2	.	DPF5 - DPF35	-4.3493	0.321	16.1	-13.548	**<0.001**
PCB 2	.	DPF15 - DPF25	-1.9883	0.183	386.5	-10.854	**<0.001**
PCB 2	.	DPF15 - DPF35	-3.1442	0.194	394.3	-16.179	**<0.001**
PCB 2	.	DPF25 - DPF35	-1.1558	0.195	392.6	-5.913	**<0.001**
PCB 3	.	DPF5 - DPF15	-1.2809	0.317	15.3	-4.045	**0.0031**
PCB 3	.	DPF5 - DPF25	-3.2508	0.334	17.6	-9.740	**<0.001**
PCB 3	.	DPF5 - DPF35	-3.1019	0.372	21.3	-8.340	**<0.001**
PCB 3	.	DPF15 - DPF25	-1.9699	0.218	404.3	-9.057	**<0.001**
PCB 3	.	DPF15 - DPF35	-1.8210	0.272	250.3	-6.697	**<0.001**
PCB 3	.	DPF25 - DPF35	0.1489	0.280	321.6	0.532	0.8503
PCB 4	.	DPF5 - DPF15	-0.7693	0.369	21.9	-2.084	0.1124
PCB 4	.	DPF5 - DPF25	-3.7523	0.493	43.2	-7.617	**<0.001**
PCB 4	.	DPF15 - DPF25	-2.9830	0.433	376.9	-6.893	**<0.001**
.	5	Methanol-PCB 1	0.1148	0.382	11.1	0.301	0.9737
.	5	Methanol-PCB 2	0.0892	0.382	11.1	0.234	0.9892
.	5	Methanol-PCB 3	0.1044	0.382	11.1	0.273	0.9737
.	5	Methanol-PCB 4	0.2488	0.382	11.1	0.651	0.8035
.	5	PCB 1-PCB 2	-0.0256	0.382	11.1	-0.067	1.0000
.	5	PCB 1-PCB 3	-0.0104	0.382	11.1	-0.027	1.0000
.	5	PCB 1-PCB 4	0.1340	0.382	11.1	0.351	0.9492
.	5	PCB 2-PCB 3	0.0152	0.382	11.1	0.040	1.0000
.	5	PCB 2-PCB 4	0.1596	0.382	11.1	0.418	0.9208
.	5	PCB 3-PCB 4	0.1444	0.382	11.1	0.378	0.9410
.	15	Methanol-PCB 1	-0.2150	0.225	47.5	-0.955	0.6026
.	15	Methanol-PCB 2	-0.3113	0.225	47.5	-1.383	0.3366
.	15	Methanol-PCB 3	-0.3719	0.230	50.5	-1.620	0.2364
.	15	Methanol-PCB 4	0.2841	0.298	65.2	0.954	0.6026
.	15	PCB 1-PCB 2	-0.0963	0.225	47.5	-0.428	0.9205
.	15	PCB 1-PCB 3	-0.1569	0.230	50.5	-0.683	0.7737
.	15	PCB 1- PCB 4	0.4991	0.298	65.2	1.676	0.2157
.	15	PCB 2- PCB 3	-0.0606	0.230	50.5	-0.264	0.9737
.	15	PCB 2- PCB 4	0.5955	0.298	65.2	1.999	0.1124
.	15	PCB 3- PCB 4	0.6561	0.301	67.3	2.178	0.0823
.	25	Methanol-PCB 1	-0.2083	0.244	58.6	-0.854	0.6613
.	25	Methanol-PCB 2	0.1629	0.229	50.7	0.7110	0.7641
.	25	Methanol-PCB 3	0.1208	0.255	60.4	0.474	0.8924

.	25	Methanol-PCB 4	-0.2363	0.443	105.1	-0.533	0.8503
.	25	PCB 1-PCB 2	0.3712	0.243	57.5	1.530	0.2632
.	25	PCB 1-PCB 3	0.3291	0.267	66.4	1.231	0.4211
.	25	PCB 1-PCB 4	-0.0280	0.450	107.5	-0.062	1.0000
.	25	PCB 2-PCB 3	-0.0421	0.254	59.3	-0.166	1.0000
.	25	PCB 2-PCB 4	-0.3992	0.442	104.6	-0.902	0.6298
.	25	PCB 3-PCB 4	-0.3570	0.456	106.9	-0.783	0.7092
.	35	Methanol-PCB 1	0.4582	0.289	91.6	1.588	0.2385
.	35	Methanol-PCB 2	0.1577	0.267	82.3	0.591	0.8281
.	35	Methanol-PCB 3	1.4203	0.326	80.0	4.353	**0.0001**
.	35	PCB 1-PCB 2	-0.3005	0.269	72.9	-1.117	0.4934
.	35	PCB 1-PCB 3	0.9621	0.328	73.6	2.931	**0.0121**
.	35	PCB 2-PCB 3	1.2626	0.309	66.6	4.084	**0.0004**

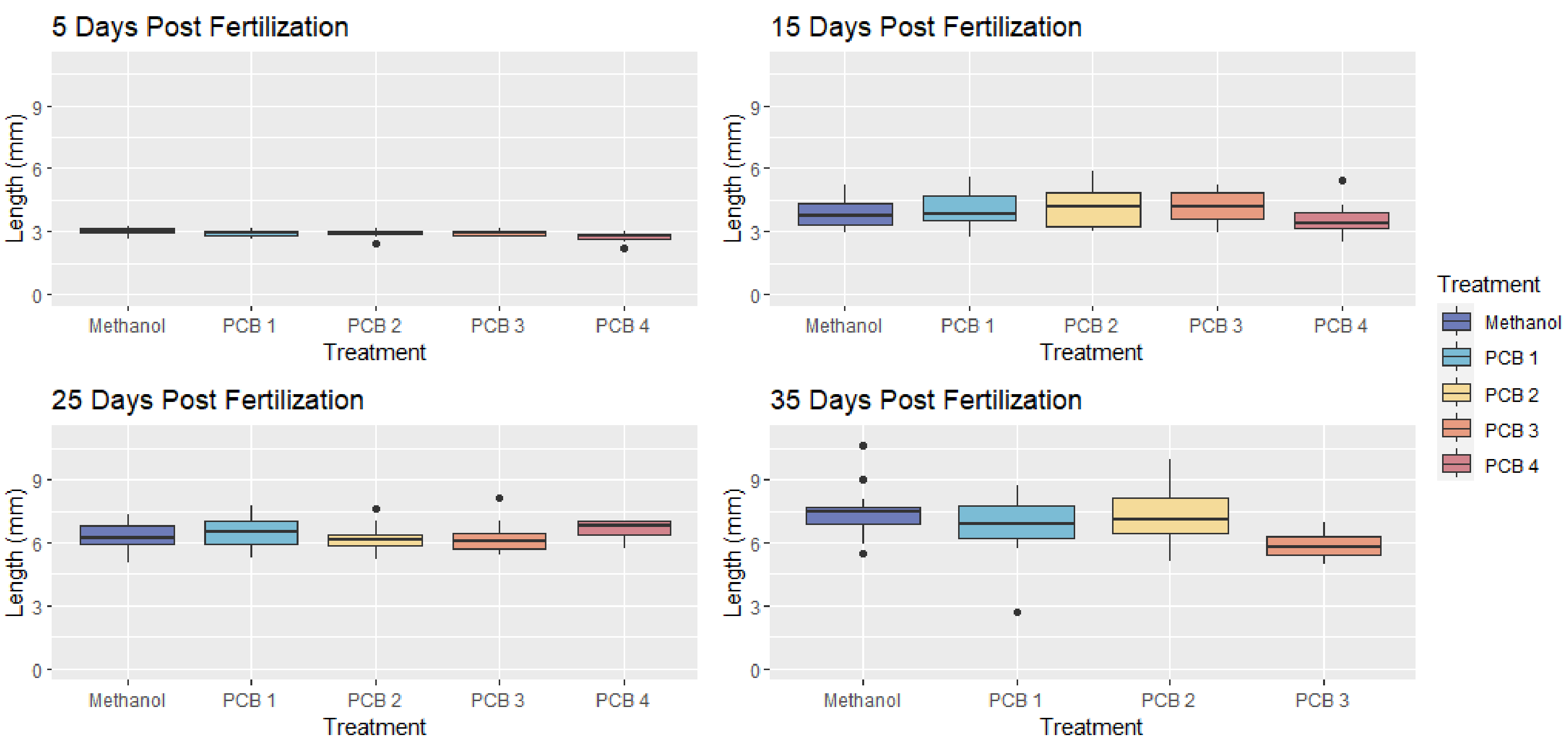

Figure S1. Boxplots Depicting Length Across Treatments.

SCRIPTS

Survival

Read in data: Daily Mortality

```
library(readxl)
KaplanMeierDailyMortality <- read_excel("Thesis/Final Data/Mortality/KaplanMeierDailyMortality.xlsx")
View(KaplanMeierDailyMortality)
DTE1 <- KaplanMeierDailyMortality
DTE1<-DTE1[which(DTE1$Treatment!=1),]
```

Set up Quantiles

```
DTE1$Quant<-NA
DTE1$Quant[which(DTE1$Treatment==2)]<-0.0
DTE1$Quant[which(DTE1$Treatment==3)]<-0.125
DTE1$Quant[which(DTE1$Treatment==4)]<-0.25
DTE1$Quant[which(DTE1$Treatment==5)]<-0.35
```

Coxph Model:

```
require(survival)
s2<-coxph(Surv(DPF,Event)~ Quant + Pop, data=DTE1)
summary(s2)
```

Metamorphosis

Read in Data: Metamorphosis

```
library(readxl)
KaplanMeierMetamorphosisNoPCB4 <- read_excel("F:/Data/Mortality/Metamorphosis/KaplanMeierMetamorphosisNoPCB4.xlsx")
View(KaplanMeierMetamorphosisNoPCB4)
DTE <- KaplanMeierMetamorphosisNoPCB4
DTE<-DTE[which(DTE$Treatment!=1),]
DTE<-DTE[which(DTE$Treatment!=6),]
DTE$Quant<-NA
DTE$Quant[which(DTE$Treatment==2)]<-0.0
DTE$Quant[which(DTE$Treatment==3)]<-0.125
DTE$Quant[which(DTE$Treatment==4)]<-0.25
DTE$Quant[which(DTE$Treatment==5)]<-0.35
```

Coxph Model:

```
require(survival)
s2<-coxph(Surv(dpf,Event)~ Quant + pop + strata(pop), data=DTE)
summary(s2)
```

Coxme Model:

```
require(coxme)
```

```
efit2<-coxme(Surv(dpf,Event) ~ Quant + pop + (1|Tank/Jar), data=DTE)
summary(efit2)
```

Feeding Efficiency

Load Packages

```
library(glmmTMB)
library(lme4)
library(MASS)
library(magrittr)
```

Read in Data: Feeding Efficiency

```
library(readxl)
FeedingTrialAll <- read_excel("Thesis/Final Data/Feeding/FeedingTrialAll.xlsx")
```

Exclude Embryo Water, Set up Quantiles

```
FeedingTrialAll <- FeedingTrialAll[which(FeedingTrialAll$Treatment!=1),]
FeedingTrialAll$Quant<-NA
FeedingTrialAll$Quant[which(FeedingTrialAll$Treatment==2)]<-0.0
FeedingTrialAll$Quant[which(FeedingTrialAll$Treatment==3)]<-0.125
FeedingTrialAll$Quant[which(FeedingTrialAll$Treatment==4)]<-0.25
FeedingTrialAll$Quant[which(FeedingTrialAll$Treatment==5)]<-0.35
FeedingTrialAll$Quant[which(FeedingTrialAll$Treatment==6)]<-0.4
```

GLMM with a Negative Binomial Distribution

```
FeedingTrial.nb <- glmer.nb(Count~ as.factor(DPF) *  as.factor(Treatment) + (1|Tank:Jar),data=FeedingTrialAll,theta=2)
summary(FeedingTrial.nb)
```

Run Pairwise Comparison

```
library(emmeans)
FeedingTrial.emm.nb <- emmeans(FeedingTrial.nb, c("Treatment","DPF"))
```

Comparisons for each feeding trial and across treatments

```
d.t2 <- pairs(emmeans(FeedingTrial.emm.nb, ~ DPF | Treatment))
t.d2 <- pairs(emmeans(FeedingTrial.emm.nb, ~ Treatment | DPF))
rbind(d.t2, t.d2, adjust = "mvt")
update(d.t2 + t.d2, adjust = "fdr")
```

Length

Read In Data: Length

```
library(readxl)
Cleansed_Lengths <- read_excel("F:/Data/Length/Cleansed_Lengths.xlsx")
View(Cleansed_Lengths)
LengthAll <- Cleansed_Lengths
```

Exclude Embryo Water & PCB 4

```
LengthAll <- LengthAll[which(LengthAll$Treatment!=1),]
LengthAll <- LengthAll[which(LengthAll$Treatment!=6),]
LengthAll$Length <- LengthAll$`Length (mm)`
```

Set up Quantiles

```
LengthAll$Quant<-NA
LengthAll$Quant[which(LengthAll$Treatment==2)]<-0.0
LengthAll$Quant[which(LengthAll$Treatment==3)]<-0.125
LengthAll$Quant[which(LengthAll$Treatment==4)]<-0.25
LengthAll$Quant[which(LengthAll$Treatment==5)]<-0.35
LengthAll$Quant[which(LengthAll$Treatment==6)]<-0.4
View(LengthAll)
```

GLMM

```
Length.a <-glmer(Length~ as.factor(DPF) *  as.factor(Treatment) + (1|Tank:Jar), data=LengthAll)
summary(Length.a)
```

Run Pairwise Comparison

```
library(emmeans)
Length.emm.a <- emmeans(Length.a, c("Treatment","DPF"))
```

Comparisons at each time point and across each treatment

```
d.t <- pairs(emmeans(Length.emm.a, ~ DPF | Treatment))
t.d <- pairs(emmeans(Length.emm.a, ~ Treatment | DPF))
rbind(d.t, t.d, adjust = "mvt")
update(d.t + t.d, adjust = "fdr")
```